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Loss of genetic diversity in isolated populations of an alpine endemic *Pilosella alpicola* subsp. *ullepitschii*: effect of long-term vicariance or long-distance dispersal?

B. Šingliarová · J. Chrtek Jr · P. Mráz

Abstract *Pilosella alpicola* subsp. *ullepitschii* (Asteraceae) is a strictly allogamous, diploid Carpathian endemic. Its distribution range comprises two areas separated by about 600 km. While in the Western Carpathians (Slovakia and Poland) the taxon occurs in numerous sites, only four localities of man-made origin are known from the Eastern and Southern Carpathians (Romania). We used allozyme markers to test two likely possible scenarios for the origin of this disjunction: long distance dispersal and vicariance. Our data indicate a significant loss of genetic diversity in the isolated Eastern and Southern Carpathian populations in following genetic parameters (averaged per region): percentage of polymorphic loci (38.9% found in the Eastern and Southern Carpathians versus 58.3% in the Western Carpathians), allelic richness (1.4 vs. 1.6), expected heterozygosity (0.134 vs. 0.235), mean number of distinguishable multilocus genotypes (4.3 vs. 10.6) and proportion of distinguishable multilocus genotypes (0.34 vs. 0.68). Higher proportion of homozygous loci found in the Eastern and Southern Carpathian populations might indicate a higher rate of inbreeding due to non-random mating. We assume that these genetically depauperate populations have experienced a very strong genetic bottleneck, probably due to a founder effect. Although our data suggest that the long-distance dispersal model is most likely, more discriminate genetic markers should be used to test this further.

Keywords Allozymes · Asteraceae · Carpathians · Bottleneck · Disjunction · Founder effect · *Hieracium* · *Pilosella alpicola* subsp. *ullepitschii*

B. Šingliarová (✉)
Institute of Botany, Slovak Academy of Sciences,
Dúbravská cesta 14, 84523 Bratislava, Slovakia
e-mail: barbora.singliarova@savba.sk

J. Chrtek Jr
Institute of Botany, Academy of Sciences of the Czech Republic,
25243 Průhonice, Czech Republic
e-mail: chrtek@ibot.cas.cz

J. Chrtek Jr
Department of Botany, Charles University, Benátská 2,
12801 Praha, Czech Republic

P. Mráz
Département de Biologie, Unité d'Ecologie & Evolution,
Université de Fribourg, 1700 Fribourg, Switzerland
e-mail: patrik.mraz@unifr.ch

Introduction

Many plant species currently have disjunct distribution occurring in two or more remote geographical areas. In general, three major processes may lead to the formation of these distributions (e.g. Raven 1972; Cox and Moore 2005; Lomolino et al. 2005): (1) Ancient tectonic events such as continental drift resulted in intercontinental splits in species ranges (Campbell 1942; Raven and Axelrod 1974); (2) Smaller scale disjunctions could be attributed to more recent climate changes (glacial-interglacial cycles during the Quaternary) when the range of a species has been fragmented by the extinction of intervening populations due to the loss of suitable habitats; survival was possible under favourable conditions only in refugia and/or isolated areas with relictual characteristics (Hewitt 1996, 2000; Comes and Kadereit 1998; Stehlik 2000, 2003; Kropf et al. 2003); (3) Long distance dispersal. While in the first two cases the disjunctions were caused by large-scale temporal or spatial changes, the latter process involves very often stochastic and singular events. Various abiotic and biotic factors such as water, wind or animals might contribute to

the long-distance dispersal at both small and large scale by dispersing plant propagules across otherwise impassable barriers (Ridley 1930; Cain et al. 2000; DeQueiroz 2005). Undoubtedly, human activities are among the most important factors driving and shaping the range of many plant species. Alien plant invasions are the best examples of the influence of man (e.g. Elton 1958; Heywood 1989).

Genetic diversity is not well understood for many species inhabiting isolated, fragmented systems. Processes leading to the range fragmentation might have strong consequences on the genetic structure and differentiation of populations in isolated areas. High mountain floras offer an excellent opportunity for inferring biogeographical processes because of the island-like pattern of their distributions. In the almost complete absence of macrofossils of mountain herbs and often the impossibility of reliably assigning pollen found in deposits to a particular species, molecular techniques provide a tool for testing the biogeographical hypotheses (e.g. Avise 2000).

The *Pilosella alpicola* group (syn. *Hieracium alpicola*; Asteraceae, Lactuceae) comprises several allopatric alpine taxa with a very polydisjunctive range across high mountains of Central and Southern Europe (Alps, Carpathians and Balkan mountains) (Fig. 1; Bräutigam 1992). At least four taxa can be clearly distinguished according to detailed morphometric study (Šingliarová et al., unpublished data). Traditionally, these taxa are treated at subspecies level (Zahn 1922–1930); however, morphological differentiation, as well as the differences in cytotype pattern, absolute genome size (Šingliarová et al., unpublished data) and their non-overlapping distribution favour species taxonomic concept. On the other hand, limited variation in ITS sequences, concerning mostly single site polymorphism, clearly shows very close relatedness of all taxa from the *P. alpicola* group (Mráz et al., unpublished data).

Pilosella alpicola subsp. *ullepitschii* (Błocki) Soják (we prefer using subspecies rank because valid combination at the rank of species within *Pilosella* still does not exist) is a Carpathian endemic, whose range is split into two disjunct areas separated from each other by a distance of about 600 km (see Fig. 1). The major area of the distribution is located in the Vysoké and Západné Tatry Mts (Western Carpathians, Slovakia and Poland), where more than 40 localities are known (based on literature and thorough herbarium revision, Šingliarová and Mráz, unpublished data). In contrast, only four isolated populations occur in the Nemira and Bucegi Mts (Romanian Eastern and Southern Carpathians, respectively) (Fig. 1) (Šingliarová and Mráz, unpublished data). Besides the difference in distribution frequency, the areas differ in ecological characteristics of the habitats. In the Western Carpathians, *P. alpicola* subsp. *ullepitschii* grows in native alpine meadows, while in the Romanian area it is confined to man-made habitats—pastures of secondary origin.

During a taxonomic and biogeographic study of the *Pilosella alpicola* group, we have investigated the origin of disjunction of this endemic subspecies coupled with very unusual ecological circumstances of the smaller Romanian area. Specifically, using co-dominant allozyme markers, we aim to test the two most probable scenarios for the origin of the geographical discontinuity, vicariance versus long distance-dispersal. The processes should result

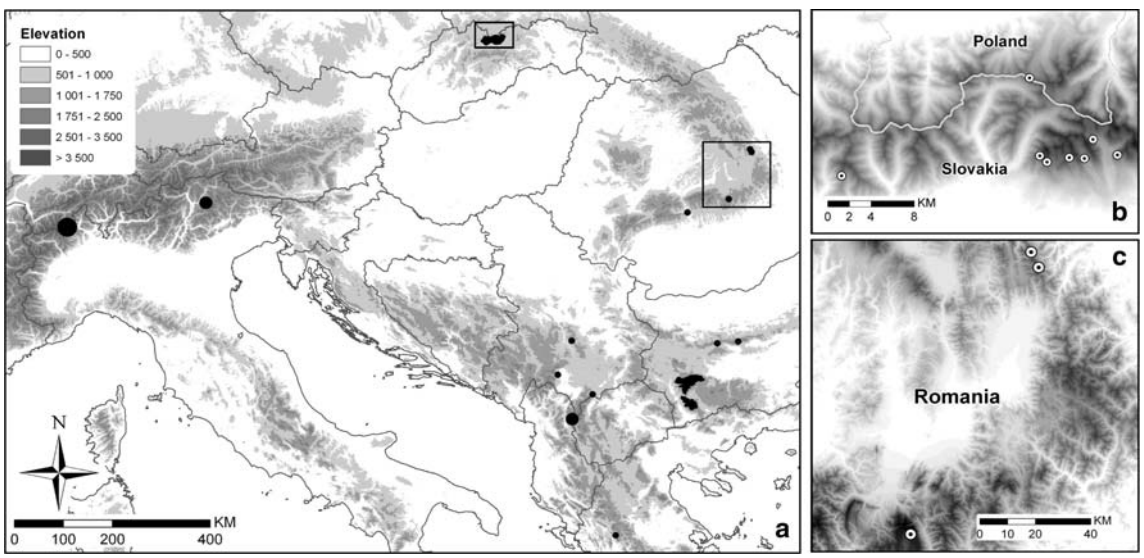

Fig. 1 Total range of the *Pilosella alpicola* group (**a**), with the range of *P. alpicola* subsp. *ullepitschii* (Błocki) Soják given in the frames. The geographical location of the 11 studied populations of *P. alpicola* subsp. *ullepitschii* in the Western Carpathians (Vysoké and Západné Tatry Mts) and the Eastern and Southern Carpathians (Nemira and Bucegi Mts) are shown in detail (**b** and **c**, respectively)

in a different genetic structure of the resident populations (e.g. Avise 2000; Kropf et al. 2006): (1) Populations resulting from vicariance should display a certain level of genetic differentiation depending on the time since the areas were isolated; furthermore, we might expect some decrease of the genetic diversity in the fewer, isolated Eastern and Southern Carpathian populations compared with the populations in the Western Carpathians due to a reduction of population size and restricted gene flow; (2) In the case of recent long-distance dispersal, the populations from Romania should be a subset of the gene pool present in the Western Carpathian group of populations, if we consider unidirectional dispersal from the Western Carpathians as a source area to the sink area in Romania. Thus, no genetic differentiation between the areas would be exhibited. However, the predicted genetic consequences resulting from vicariance and long-distance dispersal may be blurred by other factors. The most important are the differences in the rate of mutations, the number of founder events and source areas, changes in the breeding system, genetic drift, the severity of bottleneck, genetic enrichment by hybridisation, etc. (Hamrick and Godt 1989; Charlesworth et al. 1990; Leberg 1992; Charlesworth and Pannell 2001; Busch 2005; Kapralov et al. 2006). Considering the abundance of populations of *P. alpicola* subsp. *ullepitschii* in the Western Carpathians compared to the Eastern and Southern Carpathian area with its unusual habitat, the long-distance dispersal model is the most likely. On the other hand, a vicariant explanation cannot be excluded if a taxon survived in some refugial, tree-less areas in the proximity of the recent localities. Moreover, the whole *Pilosella alpicola* complex shows polydisjunctive distribution (cf. Fig. 1); such a pattern is usually explained as the result of fragmentation of a previously continous range.

Materials and methods

Taxon studied

P. alpicola subsp. *ullepitschii* is a (5–)10–20 cm tall perennial herb with rosette leaves and one to four yellow capitula. Only obligatory allogamous diploids ($2n = 2x = 18$) were found in the whole range (Šingliarová and Mráz, unpublished data). Clonal propagation is very limited in comparison with other taxa of the genus *Pilosella*. Very short (up to 3 cm) underground stolons have been observed; however, the presence of this trait is not constant in all individuals. If the stolons are present, the plants may form dense tufts of leafy rosettes up to 15 cm in diameter. Long-distance dispersal is possible via achenes with pappus.

Areas studied

The Vysoké Tatry Mts are the highest part of the Western Carpathians (and at the same time of the whole Carpathian arc), the highest peak reaching 2,655 m. There is a fully developed alpine belt from 1,800–1,900 to 2,400–2,500 m a.s.l. The Západné Tatry Mts are the continuation of the Vysoké Tatry Mts westwards, but they are lower (elevation maximum is 2,248 m), with a narrower alpine zone. Both ranges are built mainly from acidic granites. The Nemira Mts is a short flysch mountains range in the Eastern Carpathians (Romania) with highest summit reaching 1,649 m. The highest elevation represents a supramontane belt (the alpine and natural subalpine levels are missing) covered predominantly by spruce forest. Secondary grasslands in the spruce belt were created for pastures during medieval times. In addition to the intensively grazed pastures, small flysch rocks are scattered in the summit region. The southernmost population of *P. alpicola* subsp. *ullepitschii* is located in the Bucegi Mts (Southern Carpathians, Romania) reaching 2,505 m a.s.l. The only known locality is situated in the large deforested plateau (2,200–2,300 m) composed of calcium-rich conglomerates. On the plateau, the presence of *Pinus mugo* communities as the potentially natural vegetation has been suggested (Beldie 1940). The area is heavily affected by tourism (lifts, access roads) and overgrazing.

Sampling

Plants were collected from their natural populations in the summers of 2005–2007 and transferred to the experimental garden of the Institute of Botany, Slovak Academy of Sciences, Bratislava. Eight populations of *P. alpicola* subsp. *ullepitschii* from the Western Carpathians, two from the Eastern and one from the Southern Carpathians were sampled (Table 2, Fig. 1). Individuals were sampled usually 1–10 m from each other depending on the size and density of the population. The population size (number of flowering plants) was estimated and designated as follows: small ($n < 50$), medium ($n < 200$) and large ($n > 200$). In spite of our effort to include an equal number of individuals per population for isozyme analysis, plant numbers were finally uneven, because several plants died either during transport or in early stage of cultivation. Therefore, 11–17 plants per sampling site were used for allozyme analysis. In total, allozymes of 166 plants were analysed.

DNA ploidy level of all analysed plants was confirmed by flow cytometry using propidium iodide staining (FACSCalibur instrument Becton Dickinson, USA). In addition, exact chromosome numbers were ascertained for several plants using classical karyological method (Šingliarová and Mráz, unpublished data).

Allozyme electrophoresis

Young leaves of cultivated plants were used for allozyme extraction. Approximately 1 cm^2 (400 mg) of fresh leaf tissue was ground in an extraction buffer following Kato (1987) with some modifications: 0.1 M Tris–HCl (pH = 8.0), 70 mM mercaptoethanol, 26 mM sodium metabisulphite, 11 mM L-ascorbic acid, 4% soluble PVP, the pH adjusted after the addition of the ascorbate. Crude homogenates were centrifuged at 15,000 rpm for 10 min. Supernatants were stored at −75°C or immediately loaded onto gels. Electrophoresis (PAGE) was carried out using 8.16% separation polyacrylamid gel with the buffer 1.82 M Tris–HCl, pH 8.9; 4% stacking gel with the buffer (0.069 M Tris–HCl, pH 6.9) and electrode buffer (0.02 M Tris, 0.24 glycine, pH 8.3). The staining procedures followed Vallejos (1983) and Wendel and Weeden (1989), with some modifications (Krahulec et al. 2004; Peckert et al. 2005).

PAGE was initially performed on six isozyme systems, of which four turned out to be polymorphic and interpretable (abbreviation of the system is given at the end of the system name and its EC number is given within parentheses): phosphoglukomutase PGM (EC 5.4.2.2.), shikimate dehydrogenase SHDH (EC 1.1.1.25), 6-phosphogluconate dehydrogenase 6-PGDH (EC 1.1.1.44), superoxide dismutase SOD (EC 1.15.1.1). Dihydrolipoamiddehydrogenase DIA (EC 1.6.4.3) was not interpretable. Leucine aminopeptidase LAP (EC 3.4.11.1) had a clear and interpretable pattern in all plants except those from the Eastern and Southern Carpathians, where only a strong and consistent smear was observed.

Two enzymes systems 6-PGDH and PGM displayed two zones of activity, interpreted as two putative loci (e.g. *6-Pgdh-1*, *2*, with "1" coding for the faster locus). Banding patterns were interpreted allelically, based on allozyme patterns obtained within the larger study of *Pilosella alpicola* group including other diploid and polyploid taxa (Šingliarová et al., unpublished data). Alleles were designated sequentially with the most anodally migrating one coded as "a". Allozymes, as codominant markers, have been previously used in the genus *Pilosella* to study genetic structure, clonal reproduction and species relationships (Krahulec et al. 2004; Kashin et al. 2005; Peckert et al. 2005; Tyler 2005; Bruun et al. 2007).

Data analysis

Genetic diversity measures: mean number of alleles per locus (A), the percentage of polymorphic loci (P measured as the fraction of loci where the most common allele has a frequency <0.99), observed heterozygosity (H_o) and expected heterozygosity (H_e) were estimated using the program POPGENE version 1.32 (Yeh et al. 1999). Nei (1978) unbiased genetic distance was calculated between all populations and used to construct a UPGMA phenogram. The number of genotypes, "clonal diversity" (number of genotypes to number of samples) and number of unique genotypes were computed for each population. In addition, the level of inbreeding within populations (F_{IS}) and the mean fixation index (F) were generated for each population. The chi-square test was used to evaluate the deviations of F from zero and to test deviations from Hardy–Weinberg equilibrium. Two-sample t test was used to test the differences in genetic parameters between populations that originated from two areas: the Western and Eastern and Southern Carpathians (Table 1). Normal distribution of tested values was verified by Shapiro–Wilk test (all basic statistical tests implemented in software R cran, R Development Core Team 2006). Genetic structure was investigated using Nei's measures of genetic diversity (Nei 1987), i.e. total genetic diversity (H_T), mean genetic diversity within population (H_S) and the proportion of genetic diversity occurring among populations [$G_{ST} = (H_T - H_S)/H_T$)]. Weir and Cockerham's estimates (Weir and Cockerham 1984) of Wright's F-statistics (Wright 1965) were generated for each polymorphic locus (*Shdh-1*, *Pgm-1*, *Pgm-2*, *6Pgdh-1*, *6Pgdh-2*, *Sod-1*). Both Nei's and Weir and Cockerham's statistics were also computed separately for a set of populations from (1) the Western Carpathians and (2) the Eastern and Southern Carpathians. The average gene flow among populations (N_m) within a province was estimated following Crow and Aoki (1984). Probability values for differences between provinces are given for the two-sided t test after 10,000 permutations. All analyses were performed with FSTAT software (Goudet 1995) using the rarefaction method to minimise error due to uneven sampling size per population. Mantel test and reduced major axis (RMA) regression were conducted on natural log-transformed data of genetic distances (F_{ST})/genetic similarities calculated according to Slatkin (1993) and geographic distances to assess the model of isolation-by-distance. Tests were performed with the entire data set and for groups of populations from the Western Carpathians and the Eastern and Southern Carpathians separately, using programme IBD version 1.2 with 10,000 randomisations (Jensen et al. 2005).

Results

Variation in genetic parameters at population and regional level

Four enzymatic systems (SHDH, PGM, 6PGDH, SOD) were consistently resolved and scored for six polymorphic

Table 1 Summary genetic diversity within 11 populations of *Pilosella alpicola* subsp. *ullepitschii* (Błocki) Soják based on six putative loci

	Code	Locality	Altitude	Longitude N	Latitude E	Size	*N*	*P*	*A*	$\sum a$	*G*	*G*/*N*	G_{uni}	H_o	H_e	F_{IS}	*F*
Western Carpathians	LAL	Západné Tatry Mts, Ľaliové sedlo seddle	1,952 m	49°13′35″	19°59′30″	L	17	50	1.602	9	9	0.58	5	0.206	0.176	−0.139	0.169
	BAR	Západné Tatry Mts, Mt. Baranec	1,885 m	49°09′47″	19°44′04″	M	14	50	1.5	9	7	0.5	2	0.274	0.202	−0.323	0.355
	MLY	Vysoké Tatry Mts, Mlynická dolina valley	1,675 m	49°09′00″	20°02′48″	S	16	83.33	1.833	10	10	0.63	2	0.406	0.304	−0.306	0.334
	FUR	Vysoké Tatry Mts, Furkotská dolina valley	1,900 m	49°09′10″	20°01′40″	M	16	66.67	1.663	10	14	0.88	0	0.292	0.272	−0.04	0.074
	KRI25	Vysoké Tatry Mts, SW foothills of Mt Kriváň	1,900 m	49°09′27″	19°59′25″	S	16	66.67	1.667	9	13	0.81	4	0.292	0.281	−0.007	0.040
	MENG	Vysoké Tatry Mts, Mengusovská dolina valley	1,800 m	49°09′57″	20°03′40″	S	16	50	1.5	10	9	0.56	0	0.186	0.169	−0.07	0.100
	KRI55	Vysoké Tatry Mts, SE foothills of Mt Kriváň	1,900 m	49°09′05″	19°59′55″	M	17	50	1.5	9	12	0.71	0	0.265	0.243	−0.061	0.092
	OST	Vysoké Tatry Mts, Mt Ostrva	1,959 m	49°08′58″	20°05′22″	M	15	50	1.5	9	11	0.73	0	0.244	0.232	−0.02	0.054
	Mean							58.33	1.596	9.375	10.63	0.68		0.271	0.235	−0.121	0.152
E and S Carpathians	SAN	Nemira Mts, Mt Sandru Mare	1,640 m	46°11′57″	26°20′21″	S	14	33.33	1.332	8	3	0.21	1	0.179	0.114	−0.548	0.574
	NMA	Nemira Mts, Mt Nemira Mare	1,641 m	46°15′21.5″	26°19′25.5″	S	11	50	1.5	9	5	0.46	2	0.212	0.165	−0.239	0.283
	BUC	Bucegi Mts, Mt Caraiman	2,204 m	45°24′29″	25°28′22″	L	14	33.33	1.333	8	5	0.36	1	0.143	0.124	−0.114	0.151
	Mean							38.89*	1.388*	8.333*	4.33**	0.34**		0.178	0.134**	−0.300	0.336
Whole range	Mean							48.61	1.492	8.854	7.48	0.51		0.225	0.185	−0.211	0.244

N number of plants, *P* percentage of polymorphic loci (99% cutoff), *A* average number of alleles per polymorphic locus, $\sum a$ sum of alleles for overall polymorphic loci, *G* number of different multilocus genotypes, *G*/*N* "clonal diversity," G_{uni} number of unique multilocus genotypes, H_o observed heterozygosity, H_e expected heterozygosity (Nei 1987), F_{IS} Wright's fixation index per population over loci, *F* mean fixation index (Weir 1990)

Population size (number of flowering plants) was estimated and designated as follows: *S* small ($n < 50$), *M* medium ($n < 200$), *L* large ($n > 200$)

Statistically significant differences in mean values of genetic parameters between populations from two ranges (Western Carpathians, and Eastern and Southern Carpathians) are denoted by the following symbols: * $P < 0.05$, ** $P < 0.01$ (two-sample *t* test, see "Materials and methods")

loci (*Shdh-1*, *Pgm-1*, *Pgm-2*, *6Pgdh-1*, *6Pgdh-2*, *Sod-1*). In total, we found 14 alleles and the number of alleles per locus was either two or three. Considerable variation was observed in allelic frequencies between different populations and geographic regions (Western Carpathians vs. Southern and Eastern Carpathians) (Tables 1, 2). As a rule, the Western Carpathian populations exhibit higher allelic richness per locus (1.50–1.83) and percentage of polymorphic loci (50–83.33%), in comparison with the isolated populations from the Eastern and Southern Carpathians (1.33–1.50 and 33.33–50%, respectively). Similarly, the proportion of distinguishable multilocus allozyme genotypes to the number of individuals per population ("clonal diversity") was obviously higher in the Western Carpathians (0.50–0.88) than in the Eastern and Southern Carpathians (0.21–0.46). A comparison of observed (H_o) and expected heterozygosity (H_e) revealed an excess of heterozygotes ($F_{IS} < 0$) in all populations (Table 1); however, only in one population (SAN), this excess was significant from zero. One population (MLY, from the Western Carpathians) showed deviation from Hardy–Weinberg equilibrium (at significance level 0.05, $P = 0.0137$).

Hierarchical distribution of genetic diversity

Estimates of the total genetic diversity (H_T) averaged over the six variable loci reached a mean value of 0.257 (ranging from 0.033 to 0.485), and within-population genetic diversity (H_S) reached a mean value of 0.214 (range 0.028–0.397) (Table 3). The between-population component of diversity (G_{ST}) ranged from 0.132 to 0.188, with mean value of 0.168 (Table 3).

Genetic differentiation between areas

Because of the striking disparity in intrapopulation values of genetic diversity observed between the populations from two remote areas (Table 1), we divided the populations into two groups corresponding to their geographic origin. Three unique alleles were recorded in the Western Carpathians and two unique alleles in the Eastern Carpathians—in the Nemira Mts. The *Shdh-1-b* allele was present in all Western Carpathian populations, while the *6-Pgdh-2-e* allele was observed in six plants from MLY population, and *Shdh-1-a* in only one plant from LAL population (all from the Western Carpathians). Each of two Nemira populations harbours one unique allele: *Shdh-1-d* was observed in three plants in SAN and *Pgm-1-b* in four plants from NEM. Altogether, nine alleles were shared between the two regions (Table 2). Total genetic diversity (H_T) was higher in the Western Carpathians in comparison to the Eastern and Southern Carpathians. Most of the diversity was explained by differences between individuals within populations in the Western Carparthian group ($H_S = 0.242$), while only 6% of the diversity could be attributed to

Table 2 Estimated allele frequencies at six polymorphic loci in 11 populations of *Pilosella alpicola* subsp. *ullepitschii* (Błocki) Soják

Population/locus	Allele	MLY	FUR	LAL	BAR	KR25	MEN	KR55	OST	SAN	NMA	BUC
N		16	16	17	14	16	16	17	15	14	11	14
Shdh-1	a	–	–	0.03	–	–	–	–	–	–	–	–
	b	0.22	0.41	0.59	0.5	0.53	0.21	0.44	0.43	–	–	–
	c	0.78	0.59	0.38	0.5	0.47	0.79	0.56	0.57	0.89	1	1
	d	–	–	–	–	–	–	–	–	0.11	–	–
Pgm–1	a	1	1	1	1	1	1	1	1	1	0.82	1
	b	–	–	–	–	–	–	–	–	–	0.18	–
Pgm-2	a	0.25	0.47	0.09	0.21	0.31	0.15	0.47	0.3	0.43	1	0.5
	b	0.75	0.53	0.91	0.79	0.69	0.85	0.53	0.3	0.67	–	0.5
6–Pgdh-1	a	0.25	0.09	–	–	0.56	–	–	–	–	–	–
	b	0.75	0.91	1	1	0.84	1	1	1	1	1	1
6–Pgdh-2	a	–	–	–	–	–	–	–	–	–	–	–
	b	–	–	–	–	–	–	–	–	–	–	–
	c	0.81	1	1	1	1	1	1	1	1	1	1
	d	–	–	–	–	–	–	–	–	–	–	–
	e	0.19	–	–	–	–	–	–	–	–	–	–
Sod-1	a	0.31	0.41	0.74	0.25	0.56	0.32	0.35	0.4	–	0.27	0.86
	b	0.69	0.59	0.36	0.75	0.44	0.68	0.65	0.6	1	0.73	0.14

The codes for the populations follow those given in Table 1

Allele absent in a particular population is marked as "–"

Table 3 Genetic variation and structure for six polymorphic loci in 11 *Pilosella alpicola* subsp. *ullepitschii* (Błocki) Soják populations

Locus/ parameter	Nei's			Wright's			
	H_S	H_T	G_{ST}	F_{IT}	F_{ST}	F_{IS}	N_m
Shdh-1	0.359	0.438	0.181	0.120	0.179***	−0.072	1.024
Pgm-1	0.028	0.033	0.147	0.005	0.164***	−0.190	1.138
Pgm-2	0.397	0.464	0.143	−0.041	0.139***	−0.209	1.383
6-Pgdh-1	0.076	0.087	0.132	−0.035	0.141***	−0.204	1.360
6-Pgdh-2	0.028	0.034	0.152	−0.001	0.165***	−0.198	1.130
Sod-1	0.393	0.485	0.188	0.094	0.194***	−0.124	0.927
Overall	0.214	0.257	0.168	0.051	0.169	−0.142	1.010

H_T total genetic diversity of the species, H_S mean within-population genetic diversity, G_{ST} proportion of the total genetic diversity among populations

F_{IT}, F_{ST}, F_{IS} = Weir and Cockerham (1984) estimates of Wright's F statistics

*** F significant deviation $P < 0.001$ from the null expectation of $F = 0$

between-population component ($G_{ST} = 0.064$) (Table 4). Conversely, Eastern and Southern Carpathian populations displayed a much lower value of within-population genetic diversity ($H_S = 0.138$), and 28% of diversity is due to between-population differences ($G_{ST} = 0.277$) (Table 4). Considerably higher values of F_{ST}, and thus strong differentiation among isolated populations, was found in Romanian Carpathians, while significantly lower values were observed in continuous range in the Western Carpathians (Table 4). Significant differences in allelic richness, total genetic diversity and estimate of population subdivision (F_{ST}) were detected between two areas (Table 5). Although the differences in observed heterozygosity and inbreeding coefficient were not statistically significant, some obvious trends can be suggested (Table 5). The estimated level of gene flow among three populations in Romania was extremely low ($N_m = 0.181$). On the other hand, effective gene flow was ascertained among populations from the Western Carpathians ($N_m = 2.36$).

The Mantel test revealed a significant correlation between the genetic and geographic distances in the whole range ($r = 0.666$, $R^2 = 0.304$, $P = 0.008$). When testing this correlation in two regions separately, we obtained different results. No significant association was found in the Western Carpathians ($r = 0.036$, $R^2 = 0.003$, $P = 0.394$), while the correlation was very strong in the Eastern and Southern Carpathians ($r = 0.782$, $R^2 = 0.612$), however, without statistical support ($P = 0.334$), most likely due to a small number of populations ($N = 3$).

Cluster analysis (UPGMA) based on Nei's genetic distance showed two main clusters matching the geographical origin of populations: the Western Carpathians and the Nemira Mts (Eastern Carpathians), with the exception of the placement of the Bucegi population (Southern Carpathians), which shows affinities to the Western Carpathian group (Fig. 2).

Table 4 Nei's and Wright's statistics and estimates of N_m per provinces of *Pilosella alpicola* subsp. *ullepitschii* (Błocki) Soják from the Western Carpathians (populations 1–8) and the Eastern and Southern Carpathians (populations 9–11)

Region	Nei's			Wright's			
	H_T	H_S	G_{ST}	F_{IT}	F_{IS}	F_{ST}	N_m
W Carpathians	0.258	0.242	0.064	−0.034	−0.118	0.075	2.36
E and S Carpathians	0.191	0.138	0.277	0.199	−0.292	0.380	0.181

For definitions of the symbols used, see Table 3

Table 5 Comparison of population polymorphism of *Pilosella alpicola* subsp. *ullepitschii* (Błocki) Soják between the Western Carpathians (eight populations) and Romanian Eastern and Southern Carpathians (three populations)

Diversity measure	Western Carpathians	Romanian E and S Carpathians	P
A	1.595	1.383	0.04080
H_o	0.270	0.175	0.06430
H_S	0.241	0.136	0.01240
F_{ST}	0.075	0.380	0.02530
F_{IS}	−0.118	−0.292	0.0746

A average number of alleles per polymorphic locus, H_o observed heterozygosity, H_S gene diversity

F_{ST} and F_{IS} were estimated following Weir and Cockerham (1984)

Probability values (P) are provided for two-sided t test after 10,000 permutations

Discussion

Genetic diversity in major and isolated areas

The isolated and less numerous Eastern and Southern Carpathian populations of *P. alpicola* subsp. *ullepitschii* exhibited a much lower level of genetic diversity than populations from the Western Carpathians representing the core area of distribution. In general, genetic diversity is expected to decrease in small and isolated populations as a consequence of processes such as genetic drift, population bottlenecks, inbreeding and founder effects (Wright 1931; Nei 1975; Hamrick and Godt 1989; Barrett and Kohn 1991; Ellstrand and Elam 1993; Schaal and Leverich 1996; Young et al. 1996).

Genetic differentiation is substantially higher among three isolated Eastern and Southern Carpathian populations ($F_{ST} = 0.38$) compared to those from the Western

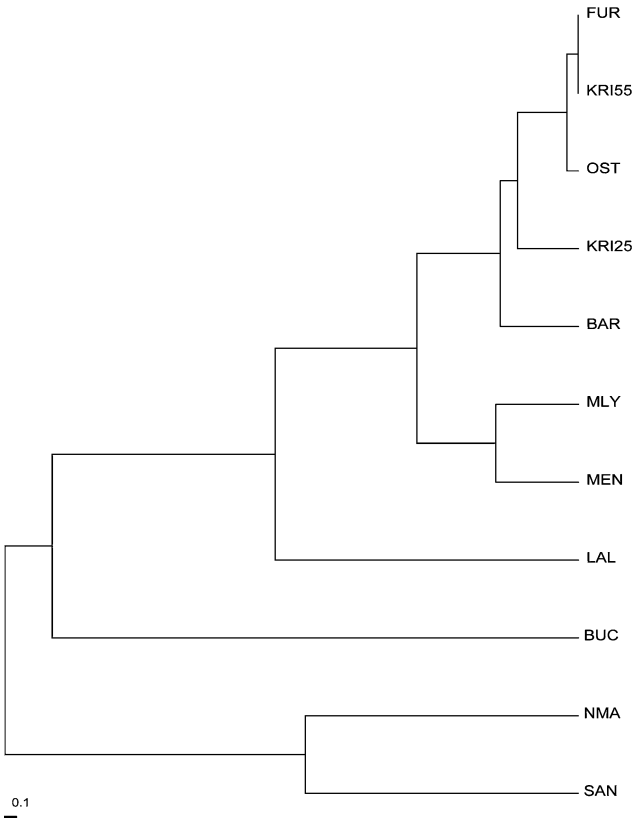

Fig. 2 Phenogram derived from a matrix of Nei (1978) unbiased genetic distances (calculated on the basis of four enzymatic systems—PGM, SHDH, 6-PGDH, SOD) following UPGMA cluster analysis for 11 populations of *Pilosella alpicola* subsp. *ullepitschii* (Błocki) Soják from the Western, Eastern and Southern Carpathians. Population designations are those given in Table 1

Carpathians ($F_{ST} = 0.075$). The higher differentiation found among Eastern and Southern Carpathian populations is not surprising considering the fact that Romanian area is composed of two relatively distant mountain ranges: one population from the Bucegi Mts is separated from three populations in the Nemira Mts by about 100 km disjunction. The pairwise F_{ST} values (data not presented, generated by IBD and used in Mantel test) indicate that the two most remote populations from the Western Carpathians separated by 27 km (BAR–OST, $F_{ST} = -0.004$) are more similar to each other than the two closest Nemira populations separated by 6 km (SAN and NMA, $F_{ST} = 0.23$). In respect of the possibility of this taxon for long distance dispersal by wind (achenes with pappus), the strong genetic isolation of the two geographically very close Nemira populations is however rather surprising. In the core area of *P. alpicola* subsp. *ullepitschii* in the Western Carpathians, more than 40 documented localities are known (Šingliarová and Mráz, unpublished data). However, according to our field observations, it is likely that this taxon occurs also in many other localities, i.e., remote and very exposed sites that are not usually visited by botanists. Thus, we can assume that numerous Western Carpathian localities form one continuous panmictic population with very effective gene flow ($N_m = 2.36$), without obvious geographic barriers through the alpine belt. On the other hand, the fragmented Nemira's populations occurring in very restricted summit areas of the main ridge are in fact separated by spruce forests and secondary pastures. Indeed, the physical barriers might likely explain a low level of gene flow ($N_m = 0.181$) in the Nemira Mts.

Another parameter notably varying between the regions is the ratio of distinguishable multilocus genotypes (*G*/*N*)—a clonal diversity index. Low level of genotype variation found consistently in the Eastern and Southern Carpathian populations could be linked with intitially low number of different genotypes funding these populations (founder effect), and/or a selective pressure might contribute to higher frequency of identical multilocus allozyme genotypes. Interestingly, the low number of distinguishable multilocus genotypes in these populations is coupled with high proportion of homozygous loci. The greater level of homozygosity found in Eastern and Southern Carpathian populations compared to those of the Western Carpathians might suggest an important role of inbreeding among genetically very close individuals, as selfing was not proved experimentally in this taxon (Šingliarová and Mráz, unpublished data). A high rate of inbreeding due to non-random mating has been suggested in small isolated populations, where the mating interactions are quite limited due to a small number of individuals (Selander 1983; Ellstrand and Elam 1993). Although the two Nemira populations are indeed small, the BUC population is probably the largest one in the whole range, consisting of several hundred plants (rough estimation). However, also in this case, we cannot exclude the possibility that BUC population, in spite of the present size, has experienced a serious bottleneck in the past due to either considerable population size reduction or a founder effect. Excess of heterozygots (negative F_{IS} values) was observed in all populations, with higher average value in the Eastern and Southern Carpathian ones. This could be a consequence of obligate outcrossing (taxon is strictly self-incompatible), or an effect of selection favouring heterozygotes (heterozygote advantage).

Origin of highly disjunctive range of *P. alpicola* subsp. *ullepitschii*

Two scenarios explaining the disjunctive range of *P. alpicola* subsp. *ullepitschii* should be concerned, namely (1) vicariance and (2) long-distance dispersal. Fragmentation of the geographic range caused by Holocene climatic changes seems to be rather common in European high

mountain species, and this vicariance model can also most likely explain the recent polydisjunct distribution of *P. alpicola* s.l. (Fig. 1). Long distance dispersal is considered to be a rarer event, although of great biogeographic importance (DeQueiroz 2005; Piñeiro et al. 2007). The Nemira populations (NEM, SAN) of *P. alpicola* subsp. *ullepitschii* are confined to lower altitudes (spruce and dwarf-pine belts) and habitats at least partly created and maintained through human activity. It is very unlikely that this heliophilous alpine taxon could have survived Holocene climatic optimum under woods in the Nemira Mts. Occurrence of other high mountain species such as *Festuca supina* and *Plantago alpina* is also closely linked with human activities and they often occur on secondary pastures at low altitudes in other mountain ranges in Romania. In the Bucegi Mts, the potential vegetation of a large and heavily deforested plateau (2,100–2,300 m) is supposed to be formed by *Pinus mugo* communities (Beldie 1940). Thus, also in this case, this subspecies does not occur in its typical habitat as in the Western Carpathians. *Pilosella alpicola* subsp. *ullepitschii* is absent from other Romanian mountain ranges, where its occurrence would be much more probable than in the Bucegi or Nemira Mts. Well developed alpine belts on granite bedrock, representing a similar kind of habitat to those from the Western Carpathians, are present at least in the Retezat Mts, Făgăraş Mts, Paring Mts, Şureanu Mts and Rodna Mts. One could argue that the present Romanian localities might have originated from the ecologically more suitable ranges mentioned above. If so, the question of why *P. alpicola* subsp. *ullepitschii* did not survive in these possible Romanian refugia with certainly more suitable habitats would be appropriate.

From the genetic point of view, the biogeographic long term vicariance model supposes a more or less comparable degree of genetic diversity in disjunctive areas and some level of differentiation among them (e.g. Thompson 1999; Avise 2000; Sanmartín et al. 2001; Kropf et al. 2006). Romanian populations are much less variable and only partly differentiated from the Western Carpathian ones. The population from the Bucegi Mts (BUC) is a genetic subset of both Western Carpatian and Nemira genetic pools without any private or region-specific allele detected, and fits all the parameters of population recently established by a few propagules (Leberg 1992). On the other hand, two private alleles observed in Nemira Mts (SAN, NMA) indicate a certain level of differentiation. This might suggest a long-term isolation with sufficient time for accumulation of mutations. The Nemira's populations are very specific in respect to small population size and restricted gene flow. In such circumstances, each new mutation, though very rare, might be fixed much more easily and rapidly than in a large panmictic population (Murawski and Hamrick 1990; Campbell and Husband 2005). Another possibility for acquiring new alleles is through introgression. Although *P. alpicola* subsp. *ullepitschii* is only alpine representative of the genus *Pilosella* in the Carpathians; in lower elevations, it can come in contact with some other taxa of the genus. In Nemira Mts, we have indeed observed its rare co-occurrence with *Pilosella lactucella* and *P. officinarum*. However, if such genetic enrichment from other taxon in *P. alpicola* subsp. *ullepitschii* was the case, it was not apparent from the morphology of the plants analysed.

Most of genetic parameters and ecological circumstances argue against a vicariance model of disjunction of Eastern and Southern Carpathian populations. An alternative explanation of a recent founder effect via long-distance dispersal should be considered. However, according to the general low-level dispersal of this taxon and also in respect to the strange habitat, natural dispersal from the Western Carpathians seems to be not very probable. The man-made, ecologically unusual habitats of the Eastern and Southern Carpathian populations might lead to the hypothesis that it could be a human introduction, presumably an unintentional one, because the plant is not very decorative or otherwise interesting. Interestingly, the only one known Romanian locality of alien species of hercynian origin with subatlantic character of distribution (cf. Ehrendorfer 1976) *Galium saxatile* L. (Rubiaceae) was found in the Nemira Mts, not far from the locality of *P. alpicola* subsp. *ullepitschii* in Mt Sandru Mare (Ciocârlan and Costea 1997). Because the bunches of the plant occur along a tourist/shepard path on the main ridge, where artificial forestation with spruce was carried out, we might assume that the presence of *G. saxatile* could be related to spruce plantation. The same explanation was proposed for introduced populations of *G. saxatile* in the Vysoké Tatry Mts (Piękoś-Mirkowa and Mirek 1978; Záhradníková and Šípošová 1982; Mirek and Piękoś-Mirkowa 1984). However, the estimated age of this artificial spruce forest on Mt Sandru Mare is about 30–40 years, while the presence of *P. alpicola* subsp. *ullepitschii* in Nemira Mts has been known at least since the beginning of the last century (the eldest voucher speciment we had at disposal came from Mt Sandru Mare from 1909).

Although our genetic, populational and ecological data favour the hypotheis of origin through long-distance dispersal, probably mediated by human activities, we have not firm evidence for that scenario. Therefore, more discriminative molecular markers should be used to test the conclusion further. We are proposing to use amplified length polymorphism (AFLPs) markers and nuclear and chloroplast DNA sequences to resolve the origin of the disjunction of *P. alpicola* subsp. *ullepitschii*. We plan also to test a possible effect on genotype selection in small isolated populations in the Nemira Mts using paternal and seed progeny analysis.

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